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# Peripheral cellular and humoral responses to infestation with the cattle tick *Rhipicephalus microplus* in Santa-Gertrudis cattle

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Abstract

Resistance to cattle tick infestation in single-host ticks is primarily manifested against the larval stage and results in the immature tick failing to attach successfully and obtain a meal. The present study was conducted to identify immune responses that characterise the tick-resistant phenotype in cattle. Thirty-five tick-naïve Santa-Gertrudis heifers were used in this study, thirty of which were artificially infested for thirteen weeks with tick larvae while five animals remained at a tick-free quarantine property to serve as a control group. Following thirteen weeks of tick infestation, the animals in this trial exhibited highly divergent tick-resistance phenotypes. Blood samples collected throughout the trial were used to measure peripheral immune parameters: haematology, the percentage of cellular subsets comprising the peripheral blood mononuclear cell (PBMC) population, tick-specific $IgG_1$ and $IgG_2$ antibody titres, IgG1 avidity for tick antigens, and the ability of PBMC to recognise and proliferate in response to stimulation with tick antigens *in vitro*. The tick-susceptible cattle developed significantly higher tick-specific $IgG_1$ antibody titres compared to the tick-resistant animals. These results suggest that the heightened antibody response either does not play a role in resistance or might contribute to increased susceptibility to infestation.

Keywords: cattle, immune response, tick, *Rhipicephalus microplus*

## 1.0 Introduction

*Bos indicus* breeds of cattle are generally more resistant to tick infestation than *Bos taurus* breeds (1,2,3) and this is one of the reasons Brahman cattle are used extensively throughout northern Australia where *Rhipicephalus (Boophilus) microplus* is endemic. However, increasing pressure from domestic and overseas consumer markets is driving producers to introduce more *Bos taurus* genetic content into their herds due to the European breeds' superior productivity and meat quality. Composite breed animals such as the Santa-Gertrudis present an attractive alternative to pure *Bos indicus* cattle in tick-endemic regions of northern Australia due to their blend of good meat quality and reproductive traits, together with the ability to acquire high levels of tick-resistance (2,3). An alternative strategy to ensure good productive characteristics in addition to increased tick-resistance is to select for tick-resistance within pure *B. taurus* herds. A breeding program at CSIRO laboratories in Rockhampton demonstrated that high levels of tick resistance (>98%) can be achieved in pure *B. taurus* animals with the generation of the Belmont Adaptaur (4), a Hereford × Shorthorn animal intensely selected for increased tick resistance over 25 years. However, the Belmont Adaptaur did not become a commercial success and its development has ceased. The main difficulty with selecting for host resistance in cattle to ticks is that identifying highly resistant individuals using the standard tick count is not a feasible option in a commercial setting. Consequently, predictive phenotypic or genotypic markers for tick resistance would be a better approach to develop herds with high levels of tick resistance.

Cattle tick infestation induces diverse bovine physiological responses including haemostasis, inflammation, and cell mediated and humoral immunity (5). The bovine host produces antibodies against tick antigens introduced during the blood feeding process (7,8,9,10) and there is large variation in antibody levels between individuals and infestation levels (11). The number of circulating T lymphocytes and antibody response to ovalbumin injection was decreased in *B. taurus* cattle heavily infested with *R. microplus* compared to their tick-free counterparts (12). This finding implies an immunosuppressive or immunoregulatory response to tick infestation. Conversely, Rechav (13) reported that Simmentaler cattle

(*B. taurus*) heavily infested with several African tick species had higher white blood cell (WBC) counts and levels of serum gamma globulins than Brahman (*B. indicus*) cattle managed under the same conditions. Sustained heavy infestation with *R. microplus* alters host haemostatic mechanisms by inhibiting platelet aggregation and coagulation functions (14), and altering the level of acute phase proteins in the susceptible host (15).

The protein composition of tick saliva changes over the course of the blood feeding process (16,17,18) and it has been suggested that the constantly changing array of immunogens to which the host is exposed creates a complex pattern of immune reactivity (19). This constantly changing profile of proteins has made it difficult to identify those antigens produced by *R. microplus* that are responsible for initiating resistance against the cattle tick in the bovine host.

We have previously reported that tick-resistant Brahman (*B. indicus*) cattle demonstrate different peripheral cellular, humoral and white blood cell (WBC) gene expression profiles from tick-susceptible Holstein-Friesian (*B. taurus*) cattle following periods of challenge with *R. microplus* (20). It was found that after a period of artificial infestations, Brahman cattle had a significantly higher percentage of T cell subsets comprising their peripheral blood mononuclear cell compared to the Holstein-Friesian cattle, whereas the Holstein-Friesians displayed higher percentages of monocytes and MHCII-expressing cells in their peripheral circulation. These observations were supported by microarray analysis of WBC gene expression, suggesting that the Brahman animals elicited a predominantly T cell-mediated response to tick infestation, as opposed to the sustained inflammatory-type response elicited by the Holstein-Friesian animals. However, high tick-specific $IgG_1$ levels measured in the Holstein-Friesian animals suggested that these animals had also developed a T cell response to tick infestation.

Our aim was to examine the  examine the peripheral cellular and antibody responses of previously tick-naïve Santa-Gertrudis cattle to infestation with *R. microplus*. The Santa-Gertrudis is a stable composite breed composed of approximately 5/8 *B. taurus* (Shorthorn) and 3/8 *B. indicus* (Brahman), and has a wide

range of resistance to *R. microplus* infestation (2,3). This study was distinct from previously reported work in that it makes sequential observations on initially naive animals from a taurine × indicine hybrid breed of cattle with an expected range of tick resistance levels that encompasses the ranges that have been documented previously for pure indicine and taurine cattle. The aim of this study was to track the development and eventual stabilisation of the bovine immune response to *R. microplus* infestation in previously unexposed animals to identify those responses that characterise the resistant phenotype.

## 2.0 Materials and methods

### 2.1 Animals

Thirty-five Santa-Gertrudis heifers aged 12 months with no previous exposure to *R. microplus* were sourced from a tick-free region of Australia. All animals had been vaccinated against the organisms that cause tick fever in Australia, *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*, prior to the commencement of the trial. Five animals were transported directly to the Queensland Primary Industries and Fisheries' (QPIF) Tick Fever Centre (tick-free quarantine property) to serve as a tick-free control group. The remaining thirty animals were transported to the University of Queensland's Pinjarra Hills Droughtmaster Unit. The Tick Fever Centre and the Pinjarra Hills facilities are located in Brisbane, Australia, and are less than 6 km apart on opposite banks of the Brisbane River. Animals at both facilities were therefore subjected to similar environmental conditions, and both groups were run in shaded paddocks and fed on pasture supplemented with whole cotton seed for the duration of the trial. The study was conducted under approval from the University of Queensland Animal Ethics Production and Companion Animals Committee.

## 2.2 Tick infestations and animal resistance rankings

At the commencement of the trial, the 30 animals at the Pinjarra Hills facility were each artificially infested with 10 000 (0.5 g) *R. microplus* larvae applied to the neck and withers. This was repeated weekly for 13 weeks, and the animals were simultaneously exposed to ticks under natural conditions in tick-infested pastures. The larvae used to artificially infest the cattle were of the Non-Resistant Field Strain (NRFS) (21) that is maintained free of *Babesia* and *Anaplasma* organisms at the Queensland Department of Agriculture and Fisheries' Biosecurity Science Laboratories. Larvae were maintained at 28°C and approximately 95% humidity and applied to animals 7-14 days after hatching. Standard tick counts were undertaken weekly for 13 weeks as described by Utech *et al.* (2,3) to assess each animal's ability to resist infestation. Briefly, tick counts were performed by counting every tick sized between 4.5 and 8 mm on one side of the animal. Tick counts were performed at 21 days following the larval infestation. Tick count data recorded over 13 weeks were analysed using a mixed effects model fit by restricted maximum likelihood (REML) (where the fixed effect was tick count and the random effect was animal) to rank each animal on its ability to resist tick infestation. Animals were subsequently divided into resistance status groups. The 6 animals consistently identified as the most resistant animals during the 10-week period after an initial 7-week adaptation period after initial infestation were classified as 'Resistant'; the 6 animals consistently identified as being the least resistant animals during the same time period were classified as 'Susceptible,' and the rest were classified as 'Middle' (18 animals).

## 2.3 Blood sampling

Blood samples were collected from animals at the Pinjarra Hills facility prior to initial tick infestation, at 21 d post infestation 1, and then weekly for a further 9 weeks. There was one week towards the end of the trial, corresponding to 84 d post infestation 1, that blood samples were not obtained. Blood samples were collected via jugular venipuncture into 5 × 9 ml Vacuette® blood tubes (Greiner Bio-One); 2 × lithium

heparin, 2 × Z clot activator, and 1 × EDTA. Blood was simultaneously collected from the 5 uninfested control animals at the Tick Fever Centre.

### 2.4 Haematology

Haematological analysis of blood samples collected in EDTA was undertaken using a VetABC animal blood cell counter (ABX Hematologie). Haematological analysis was performed on all blood samples collected from 35 animals at both facilities (Pinjarra Hills and Tick Fever Centre).

### 2.5 Flow cytometry

Details of the methods used for cell surface staining and flow cytometric analysis have been previously described in detail (20). Briefly, whole blood collected in EDTA (100 µl) was combined with 100 µl of either a monoclonal antibody (Table 1) or isotype control (mouse IgG, Dako). The primary antibody was incubated with the blood sample for 30 min at 4°C, centrifuged to pellet the contents and the supernatant discarded. Following a wash step with phosphate buffered saline (PBS), the samples were incubated with a secondary antibody (anti-mouse IgG preadsorbed with bovine IgG conjugated to fluorescein isothiocyanate (Calbiochem) diluted 1/100 in PBS) for 30 min at 4°C. The samples were then washed and resuspended in 200 µl fixative (PBS containing 1% NaN3 and 8% formaldehyde). Flow cytometric analysis was performed on all blood samples collected from 35 animals at both facilities over the course of the trial. Results for each cellular subset are presented as a percentage of the total peripheral blood mononuclear cell (PBMC) population.

## 2.6 Tick antigen extraction

Approximately 1 000 semi-engorged NRFS female ticks (21) were collected from penned *B. taurus* cattle at QPIF for preparing tick antigen extracts from mid-gut and salivary glands as previously described (20, 22). Briefly, semi-engorged adult female ticks were dissected under a light microscope while immersed in PBS, within several hours of being removed from the host. Salivary gland and gut were removed into separate vials on dry ice and stored at -80°C prior to antigen extraction. Antigens were also prepared from whole unfed NRFS larvae (21) as previously described (20,22). Larvae were ground up using a mortar and pestle and stored at -70°C prior to antigen extraction. EDTA was added to dissected organs and ground-up tissue prior to freezing to remove divalent cations that contribute to proteolysis. The antigen extraction method employed a series of centrifugation steps to separate proteins into membrane-bound and soluble fractions. The resulting antigen extracts included: salivary gland membrane (SM), larval membrane (LM), gut membrane (GM), salivary gland soluble (SS), larval soluble (LS), and gut soluble (GS) antigen extracts. Proteolysis inhibitor (Gibco) was added to the PBS during the extraction process. Antigens were stored at -80°C until required for cell proliferation and ELISA experiments.

## 2.7 Cellular proliferation assay

The ability of peripheral blood mononuclear cells (PBMC) to proliferate in response to stimulation with tick antigen extracts *in vitro* was determined using blood collected from all animals at both facilities throughout the trial. PBMC for the proliferation assay were isolated from 18 ml blood collected in lithium heparin using the Ficoll-Paque (Pharmacia) gradient method. Cells were resuspended at $8 \times 10^6$ cells/ml in Complete Medium; RPMI-1640 medium (Sigma) that contained 10% foetal bovine serum (Invitrogen), 1% antibiotic-antimycotic (Gibco) and 2 mM L-glutamine (Gibco). The proliferation assay was performed in a 96 well flat bottom cell culture plate (Greiner-One) and assays were performed in triplicate. Each experimental well contained $4 \times 10^5$ cells with either Concanavalin-A (ConA), soluble

fractions of salivary gland (SS), mid-gut (GS) or larvae (LS), or membrane fractions of salivary gland (SM) or mid-gut (GM). ConA was diluted in Complete Medium to 5 µg/ml and dispensed at 100 µl per well. Soluble antigens SS, GS and LS were diluted to 20 µg/ml in Complete Medium, while membrane antigens SM and GM were diluted to 10 µg/ml in Complete Medium and each were dispensed at 100 µl per well. Control wells contained either media only or cells plus media, and all wells were made up to a final volume of 200 µl with Complete Medium. Cellular proliferation was measured using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics) according to the manufacturer's instructions. The mean OD of each biological sample from triplicate experimental wells was divided by the mean OD of the respective biological sample from triplicate control wells (cells plus medium) to obtain the stimulation index. Stimulation indices were employed for statistical analysis.

### 2.8 Tick-specific IgG1 and IgG2 antibody levels measured by ELISA

Serum samples obtained over the course of the trial (T0 → T91 d post-infestation 1) from resistant (n = 6), susceptible (n = 6) and tick-naive control (n = 5) animals were analysed for tick-specific $IgG_1$ and $IgG_2$ antibody levels using an indirect ELISA as previously described (20). Serum samples collected from these animals prior to tick infestation and at 21 d, 35 d, 63 d and 91d following infestation 1, were also analysed to determine the avidity of tick-specific $IgG_1$ antibodies. For the antibody avidity ELISA, experiments were performed in duplicate. One set of triplicate wells were incubated with 100 µl 8 M urea dissolved in PBS-T (PBS containing 0.05% Tween20) for 15 min following the serum incubation. The other set of triplicate wells was incubated with 100 µl PBS-T. Following this incubation, all wells were washed 6 times in PBS-T and the ELISA performed as previously described (20). The mean optical density (OD) of each biological sample from triplicate wells was calculated and the avidity index was obtained using the formula: (Urea OD / PBS-T OD) × 100 (23). Avidity indices were employed for statistical analysis.

### 2.9 Statistical analysis

A linear mixed effects model with repeated measurements (AR1 continuous correlation structure) was used to analyse the data (haematology, flow cytometry, ELISA) with the statistical computing language R (24). Fixed effects included tick count and resistance ranking (Resistant, Susceptible, Middle) and their interaction with the aim of identifying the effects of tick burden and resistance status independently. The tick-naïve control data were not included in the model but are presented in the figures to indicate either the variability of the trait in question (haematology and flow cytometry data) or to demonstrate baseline values for acquired immune response parameters (cell proliferation and ELISA data). For analysis of cell proliferation data, a one-way analysis of variance was performed on stimulation indices at each time point to determine the time points at which cellular proliferation of PBMC from tick-infested animals significantly exceeded that of the control group.

## 3.0 Results

### 3.1 Tick counts and resistance rankings

The 30 animals infested weekly with tick larvae demonstrated divergent tick counts following 13 weeks of artificial infestations. Figure 1 depicts the average tick side counts for the 6 most resistant and 6 most susceptible animals in the herd. Other animals in the herd had tick side counts intermediate to these two groups. All animals carried similar numbers of ticks on counts 1 and 2, but by count 3 (corresponding to 35 d post-infestation 1), the resistant group carried a significantly lower ($P < 0.001$) burden of ticks. Whereas tick counts of the more resistant animals tended to be lower with each successive week reflecting their developing resistance, the susceptible animals carried similar numbers of ticks throughout the trial. On the last count of adult ticks, the average number of ticks carried by the 6 most susceptible

animals in the herd was 296 (±31) per side, while the average number of ticks carried by the 6 most resistant animals in the herd was 27 (±22) ticks per side. These tick counts correspond to 88% resistant (susceptible group) and 99% resistant (resistant group) as calculated using the methods described by Utech and Wharton (2,3). Briefly, percentage resistance is calculated from the number of female ticks surviving to the semi-engorged stage as a percentage of the number of larvae originally applied in the infestation (assuming a one to one sex ratio in the larval population).

### 3.2 Haematology parameters

There was no significant difference between the groups (resistant, susceptible, middle) in haemoglobin (Hb), packed cell volume (PCV), mean cell haemoglobin concentration (MCHC), white cell count (WCC) or platelet count prior to tick infestation. Susceptible animals had a significantly lower red cell count (RCC) than middle animals (approximately $1.0 \times 10^6/mm^3$ lower than middle animals, $P < 0.05$) prior to tick infestation, and significantly higher mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) than middle animals prior to tick infestation. Susceptible animals' MCH was approximately 1.5 pg/cell higher than the middle animals ($P < 0.05$), and their MCV was approximately $4.3/\mu m^3$ higher than middle animals ($P < 0.05$).

Tick infestation was shown to significantly decrease Hb ($P < 0.01$), MCH ($P < 0.01$) and MCHC ($P < 0.05$) by an average of 0.0024 g/dl, 0.002 pg/cell and 0.0028 g/dl per tick respectively, in all animals. The model also demonstrated significant differences between groups with respect to the effect of tick infestation. Tick infestation also decreased red cell counts by $0.002 \times 10^6/mm^3$ per tick in the susceptible group ($P < 0.05$) but did not have an effect on red cell counts of other animals. Within the resistant group, every tick accounted for an increase of $0.007/\mu m^3$ in MCV ($P < 0.05$) and an increase of $0.64 \times 10^3/mm^3$ platelets ($P < 0.05$).

Figure 2(a-c) depicts the physiological effects of tick infestation on the experimental animals at Pinjarra Hills compared to their tick-naïve counterparts at the Tick Fever Centre. Red cell counts, Hb and PCV tended to increase over time in the control group, whereas these parameters in tick-infested animals remained similar to values recorded at the commencement of the trial.

### 3.3 Flow cytometry

There was no significant difference between the groups (resistant, susceptible, middle) in the percentage of $CD14^+$, $CD25^+$, $CD3^+$, $CD8^+$, $MHCII^+$, $WC1^+$ or $WC3^+$ cells comprising the PBMC population prior to tick infestation. Resistant animals had approximately 4.9% more $CD4^+$ cells than other animals prior to tick infestation ($P < 0.01$). Over all the animals, tick infestation did not have a significant effect on the percentage of $CD4^+$ cells comprising the PBMC population, however, within the resistant group every tick accounted for a decrease of 0.02% in $CD4^+$ cells ($P < 0.05$) and for susceptible animals every tick increased $CD4^+$ cells by 0.01% ($P < 0.05$).

In all animals tick infestation had a significant effect on the percentage of $CD3^+$, $MHCII^+$ and $WC3^+$ cells comprising the PBMC population; every tick decreased $CD3^+$ cells by 0.01% ($P < 0.05$), and increased $MHCII^+$ and $WC3^+$ cells by 0.02% ($P < 0.01$) and 0.01% ($P < 0.001$), respectively. However, within the susceptible group every tick accounted for a 0.02% increase in $CD3^+$ cells ($P < 0.05$). Similarly, for susceptible animals, tick infestation increased $CD25^+$ cells by 0.01% per tick ($P < 0.05$), $WC1^+$ cells by 0.007% per tick ($P < 0.05$) and $CD14^+$ cells by 0.006% per tick ($P < 0.05$). Within the resistant group every tick accounted for an increase of 0.01% in $CD14^+$ cells ($P < 0.01$) and a 0.01% decrease in $CD8^+$ cells ($P < 0.05$).

Figure 3(a-h) depicts the relative percentage of each cellular subset comprising the peripheral circulation in resistant, susceptible and control groups.

### 3.4 Cell proliferation assay

There were no differences among the three groups in the ability of their PBMC to proliferate *in vitro* in response to stimulation with ConA (Figure 4a). The proliferation of PBMC in the presence of salivary gland membrane (SM) antigen was significantly greater ($P < 0.01$) in PBMC isolated from tick-infested animals compared with the tick-naïve animals (Figure 4b) after 21 d of tick infestation (corresponding to the first maturation of adult ticks on these animals). The stimulation index of PBMC from the tick-infested groups significantly exceeded that of the tick-naïve group at 21 d post infestation 1 through to 56 d post infestation 1 ($P<0.05$). The responsiveness of cells to stimulation with SM in the tick-infested groups decreased to levels similar to the tick-naive group at 63 d post infestation 1, before increasing again at 77 d post infestation 1 ($P<0.05$). There was no proliferation of cells above background levels in response to stimulation with LM, LS, SS and GM antigen extracts (data not shown).

### 3.5 Tick specific $IgG_1$ and $IgG_2$

The susceptible animals developed significantly higher levels of $IgG_1$ antibodies specific for tick antigen extracts SM, LM, LS ($P < 0.05$) GM and SS ($P < 0.1$) than the resistant animals by the completion of the trial (Figure 5a-e). There was no significant difference between groups in antibody level prior to tick infestation. The model demonstrated a significant effect ($P < 0.001$) of tick infestation on $IgG_1$ antibody levels within the susceptible group but not in the resistant group.

The tick-infested groups presented with levels of tick-specific $IgG_1$ that were significantly higher than the tick-naïve group between 21 d and 28 d post infestation 1. There was notable variation between individuals within the susceptible group in the level of tick-specific $IgG_1$ and $IgG_2$ antibodies that developed over the course of the trial. Resistant animals did not develop $IgG_2$ levels to any of the antigen extracts that were significantly different to the tick-naive group. Two susceptible animals developed very

high tick-specific $IgG_2$ levels, 2 susceptible animals developed low-moderate $IgG_2$ levels, and 2 susceptible animals did not develop $IgG_2$ levels against all tick antigens tested (Figure 6a-e).

The susceptible animals developed $IgG_1$ antibodies that bound salivary membrane antigens with significantly higher avidity than the resistant animals by the time the trial ended after 91 d of tick infestation (Figure 7a). There was no change in the avidity of antibodies developed by either resistant or susceptible animals against salivary soluble extracts (Figure 7b), whereas the avidity of $IgG_1$ antibodies developed by the resistant group against membrane and soluble larval antigens tended to decrease over the course of the trial but this was not significant (Figure 7c-d).

## 4.0 Discussion

This manuscript describes some aspects of the evolution of the peripheral cellular and antibody response of composite breed Santa-Gertrudis cattle to *R. microplus* infestation. The cattle used in this trial had never before been exposed to *R. microplus*, and following 13 weeks of regular tick infestation in this experiment, subsequently developed divergent levels of tick resistance. Despite the divergent levels of host resistance, none of the peripheral immunological markers assessed in this study emerged as unambiguously associated with resistance or susceptibility to infestation. The most notable finding, which is consistent with our previous studies using Holstein-Friesian and Brahman cattle, was that $IgG_1$ and $IgG_2$ specific to tick antigens are consistently low in resistant cattle.

The resistance estimates reported here closely reflect those reported by Utech *et al.* (2) who undertook tick counts on two groups of Santa-Gertrudis cattle in south-east Queensland in 1978. Cattle with resistance estimates of > 98% are considered to be highly resistant to cattle ticks; 95 to 98% are considered to be moderate, 90 to 95% low and cattle with resistance < 90%, including most *B. taurus* dairy cattle, are considered to have very low resistance (3). Utech *et al.* (3) reported that the Santa-Gertrudis cattle in Queensland in 1978 had tick-resistance estimates ranging from 81.7 – 99.7% which is

similar to the range reported in the present study. This suggests that the tick-resistance status of the Santa-Gertrudis breed in Queensland has not changed significantly in the past 30 years. Lack of improvement in resistance to ticks might be a consequence of the difficulty of measuring the resistance phenotype and the relative success of acaricides in controlling tick numbersduring this period. The Santa-Gertrudis was originally chosen for use in this experiment due to its expected wide range of tick resistance. We have previously conducted comparative experiments on immune response developed against *R. microplus* by Holstein-Friesian (*B. taurus*) and Brahman (*B. indicus*) cattle (20, 25, 26). It was considered important to compare the results of these studies using only one breed, with divergent tick resistance, to eliminate any breed effect that might confound the results of the previous studies. The Santa-Gertrudis presented an ideal opportunity to examine the immune responses of tick-resistant and tick-susceptible animals, but at the same time retaining a similar genetic background against which to compare results. The tick count data reported here demonstrate that animals with high levels of tick resistance persist in herds of animals that have not been under any direct selection for tick resistance.

It is important to note that as the temporal pattern of tick burdens differed between resistant and susceptible groups over the course of the trial, the association of tick burden and immune parameters has slightly different implications within the two groups. In the resistant group tick burdens were highest at the beginning of the trial and decreased steadily throughout the trial. Therefore, if tick infestation was significantly associated with a reduction in any parameter in the resistant group, it means that the parameter would have increased throughout the trial in response to infestation and could reasonably be proposed to have a role in increasing host resistance. In the susceptible group, however, tick burdens did not systematically increase or decrease over the course of the trial, but tended to remain steady from 28 d post initial infestation until the end of the trial. Therefore, if a parameter is significantly associated with a reduction in tick burden within the susceptible group it doesn't infer that it increases over time after initial infestation and any suggestion of a potential role in increasing host resistance within this group would be somewhat weaker logically than in the resistant group.

Differences between tick-resistant and tick-susceptible Santa-Gertrudis animals in the relative percentage of cellular subsets comprising the PBMC population were not as apparent as the differences observed between the Holstein-Friesian and Brahman animals in our pilot study using previously exposed cattle (20). In that study the Brahman animals (highly resistant to tick infestation) had significantly higher percentages of $CD4^+$, $CD25^+$ and $WC1^+$ T cells than Holstein-Friesian animals (susceptible to tick infestation), which had significantly higher $CD14^+$ and $MHCII^+$ cells as a proportion of the PBMC population. These results were very similar to those subsequently reported by Dominguez et al (27) and strongly imply that more susceptible animals are characterised by more pronounced inflammation and the more resistant animals are characterised by a T regulatory response. In contrast, Carvalho et al (28) found that inflammation was associated with high levels of resistance. In the present study, tick-resistant Santa-Gertrudis animals had a significantly higher percentage of $CD4^+$ cells than other animals in the trial prior to tick infestation. Within the resistant group higher levels of $CD4^+$ were associated with lower tick burdens, reflective of results obtained in the pilot trial. However, within the susceptible group heavier tick burden was associated with increased percentage of $CD4^+$ in the PBMC population.

Consistent with the pilot trial, the tick-susceptible Santa-Gertrudis animals in the present study developed significantly higher tick-specific $IgG_1$ antibody levels against a range of tick antigen extracts compared with the resistant animals. There was a significant effect of tick infestation on increasing tick-specific $IgG_1$ in the susceptible group for all tick antigens tested, but not in the resistant group, suggesting that antibody level is a function of tick burden in susceptible animals. It was also apparent that the tick-specific $IgG_2$ response is extremely variable between individuals and if developed, was only consistently developed by animals in the tick-susceptible group in this study. This extremely variable $IgG_2$ response may contribute to the variation in antibody levels reported by other researchers measuring only tick-specific IgG such as that reported by Carvalho *et al.* (15).

Although the Ag used in the assays are not the same as those to which an animal would be exposed to in a natural infestation, the ELISA results of the present study suggests that the humoral immune response does not play an important role in bovine resistance to *R. microplus*. Tick-susceptible animals developed very high levels of $IgG_1$ antibodies specific for all tick antigen extracts, while in the resistant group, levels of $IgG_1$ antibodies tended to be lowest at the end of the trial when resistance was at its highest. Furthermore, by the end of the trial the susceptible animals had developed $IgG_1$ antibodies with a significantly higher avidity for membrane-bound salivary gland antigens than the resistant animals, whereas the avidity of antibodies from resistant animals either did not change or tended to decrease over the course of the trial. Although it is clear that there are quantitative and qualitative differences between individuals and between groups with different levels of tick resistance in the development of tick-specific antibodies (8,9,10,15), no study has been able to demonstrate that those tick antigens recognised by resistant animals confer protection or are involved in inducing a protective response. Roberts and Kerr (29) demonstrated a humoral component to tick resistance by infusing tick-naïve calves with plasma from high- and low-resistant donors and reported that a significantly lower yield of ticks was obtained from calves injected with plasma from the high-resistant donors. Although the reduction in tick yield was highly significant, the authors pointed out that the reduction only accounted for about 10% of the difference in tick burden between high-and low-resistant cattle. We reported in another study that antibody-producing plasma cells take up residence in the skin of tick-susceptible animals at the larval attachment site, perhaps assisting the tick by establishing a site of local capillary permeability (26). It may be the case in susceptible animals that the production of high levels of antibodies against a wide variety of tick antigens increases susceptibility by improving feeding success, or that recognition of immunodominant molecules in tick saliva and production of antibodies to them interferes with the development of a protective immune response.

The proliferation assay demonstrated the ability of PBMC isolated from the tick-infested cattle to proliferate in response to stimulation with membrane-bound salivary gland antigens. This response was uniformly highest at 21 days post infestation 1 and then waned towards the end of the trial. Similar to the antibody ELISA results, there was large individual variation between animals in the ability of their cells to respond to antigen extracts. The proliferative response of cells to the salivary antigen extract is a result that would be expected considering that the development of the cell-mediated response is integral to the development of the antibody response. The fact that the cell-mediated response was not detected against larval antigens, whereas the $IgG_1$ antibody response to these larval antigens was so high, is striking. Reasons for this lack of response may include the relatively low abundance of antigenic proteins in the whole larval extract.

The high levels of proliferation detected in response to the salivary gland antigens is in contrast to the other literature supporting results that suggest components of *R. microplus* saliva have an inhibitory effect on cellular proliferation *in vitro* (30-34). Many of these studies report that the addition of tick saliva to cultures reduces the ability of cells to proliferate in response to ConA stimulation. Conversely, the present results demonstrate that proliferation in the range of that expected from ConA stimulation can be achieved by stimulating the cells with salivary gland antigens alone. The differences between the results of the present study and those reported by previous researchers may be attributed to the use of whole saliva rather than the membrane bound extract used in the present study, as the soluble fraction of saliva is more likely to contain compounds such as prostaglandin-$E_2$ that has been implicated in suppressing host immune responses *in vitro* (12). Differences between the present data set and the results of others may also be attributed to the time post tick exposure that the cells are isolated from the host. Peak responses in the present study were observed at 21 days after the first infestation and tended to decrease over the course of the trial and it may therefore be the case that a proliferative response may be no longer detectable in animals that have been exposed to *R. microplus* over a long period of time.

In conclusion, the present study demonstrates that a wide range of tick-resistance capabilities exist within the Santa-Gertrudis breed. The results reiterate previous findings with respect to the pathological implications of tick infestation, particularly in heavily infested animals, demonstrated by deceased haemoglobin, mean cell haemoglobin concentration and mean corpuscular volume in tick infested animals. Tick-susceptible animals in the present study developed significantly higher levels of tick-specific $IgG_1$ antibodies compared to the resistant animals, while the development of tick-specific $IgG_2$ antibodies was variable between resistance groups and between individuals within group. These results suggest that tick-susceptible animals have the ability to recognise and respond more vigorously to tick antigens introduced via tick saliva during blood feeding and that this heightened antibody response either does not play a role in resistance or might even contribute to increased susceptibility to infestation.

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Figure Legends

Figure 1: Average weekly tick side counts for the six most susceptible (dotted line, triangle) and six most resistant (dashed line, square) animals in the herd. Results are presented as the group mean ± standard deviation from the group mean.

Figure 2(A-C): Selected haematological parameters as measured in control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) groups. Group means are presented ± standard deviation from the group mean. A: Red blood cell counts $\times 10^6/mm^3$; B: Haemoglobin g/dl; C: Packed cell volume %.

Figure 3(A-H): Relative percentage of cellular subsets comprising the peripheral circulation of control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) groups. A: $CD3^+$ T cells; B: $CD4^+$ T cells; C: $CD8^+$ cytotoxic T cells; D: $CD14^+$ monocytes; E: $CD25^+$ activated T cells; F: $MHCII^+$ cells; G: $WC1^+$ γδ T cells; H: $WC3^+$ B cells. Results are presented as the group mean with standard deviation from the group mean for each cellular subset at each sampling point.

Figure 4(A-B): Proliferation of PBMC isolated from control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) groups following stimulation with A: ConA, and B: salivary gland membrane (SM) antigen. Stimulation indices are presented as the group mean ± standard deviation from the group mean.

Figure 5(A-E): Tick specific $IgG_1$ antibody levels of control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) animals against: A: salivary membrane; B: salivary soluble; C: larval membrane; D: larval soluble; E: gut membrane antigens. Group means are presented ± standard deviation from the group mean.

Figure 6(A-E): Tick specific $IgG_2$ antibody levels of control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) animals against: A: salivary membrane; B: salivary soluble; C:larval membrane; D: larval soluble; E: gut membrane antigens. Group means are presented ±standard deviation from the group mean for control and resistant groups. Susceptible animals are depicted individually as dotted lines.

Figure 7(A-D): Antibody avidity indices for tick-specific $IgG_1$ from control (solid line, diamond), resistant (dashed line, square) and susceptible (dotted line, triangle) groups. A: salivary membrane (SM); B: salivary gland soluble (SS); C: larval membrane (LM); D: larval soluble (LS). Avidity indices are presented as the group mean percentage ± standard deviation from the group mean.

**Table 1**: Monoclonal antibodies used in flow cytometric analysis of cellular subsets.

| **Specificity** | **Identity** | **Source** | **Isotype** |
|---|---|---|---|
| Isotype control | IgG1 | Dako | IgG1 |
| CD4 | IL-A11 | Cell culture[a] | IgG2a |
| CD8 | IL-A51 | Cell culture[a] | IgG1 |
| CD14 | MM61A | VMRD[b] | IgG1 |
| CD25 (IL-2Rα) | IL-A111 | Cell culture[a] | IgG1 |
| MHCII | IL-A21 | Cell culture[a] | IgG2a |
| WC3 | CC37 | Cell culture[a] | IgG1 |
| WC1 | IL-A29 | Cell culture[a] | IgG1 |
| Goat anti-mouse | IgG-FITC | Calbiochem | IgG |

[a] Monoclonal antibodies obtained from cell culture were derived from hybridomas sourced from the International Livestock Research Institute in Kenya

[b] VMRD, Veterinary Medical Research and Development, Inc